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Mechanism of aromatic ring cleavage of β-*O*-4 lignin substructure models by lignin peroxidase

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This investigation examined the aromatic ring cleavage of β-*O*-4 lignin substructure model compounds by lignin peroxidase of *Phanerochaete chrysosporium*. Based on tracer experiments using $H_2{}^{18}O$ and $^{18}O_2$, mechanisms of the aromatic ring cleavage of the β-*O*-4 lignin models were proposed. The mechanisms involve one-electron oxidation of the β-*O*-4 lignin models by the enzyme followed by attack of nucleophiles and radical coupling with O_2.

Aromatic ring cleavage; Lignin peroxidase; Cation radical; β-*O*-4 lignin substructure; *(Phanerochaete chrysosporium)*

1. INTRODUCTION

Previous investigations showed that an extracellular lignin-degrading enzyme, lignin peroxidase (ligninase), of *Phanerochaete chrysosporium* catalyzed aromatic ring cleavage reactions of β-*O*-4 lignin substructure models [1–3] and a monomeric aromatic compound, veratryl alcohol [4]. Although the involvement of H_2O and O_2 in the aromatic ring cleavage was indicated [2,3], the mechanism of the ring cleavage has not been fully elucidated. Recently, we identified methyl muconate of arylglycerol as an immediate product of aromatic ring cleavage of a β-*O*-4 lignin substructure model dimer by the enzyme (submitted). This paper discusses, based on tracer experiments using $H_2{}^{18}O$ and $^{18}O_2$, the mechanism of the aromatic ring cleavage of β-*O*-4 lignin substructure models by the enzyme.

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2. MATERIALS AND METHODS

2.1. *Substrates and authentic compounds*

The following compounds were prepared as described previously: 1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-propane (**II**) [5]; 2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-1-methoxypropane (**II**-Me) [2]; 1,3-diethoxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (**II**-Et) [1]; 1-(4-ethoxy-3-methoxyphenyl)-glycerol β,γ-cyclic carbonate (**V**) and its acetate [6], 1-(4-ethoxy-3-methoxyphenyl)glycerol α,β-cyclic carbonate (**V′**) and its acetate [1]; acetate of 1-(4-ethoxy-3-methoxyphenyl)glycerol γ-formate (**VI**), (**VI**-Ac) [7]; methyl oxalate of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-hydroxypropane (**VII**-Et) [1]. Syntheses of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-propane (**I**-Et) and methyl *cis,cis*-muconate of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-hydroxypropane (**III**) will be reported elsewhere (submitted). ^{1}H-NMR of **I**-Et ($CDCl_3$) δ(ppm), 1.19(3H×2,t), 1.45(3H,t), 3.4–3.6(4H,m),

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3.74(3H,s), 3.75(2H), 3.82(3H,s), 4.08(2H,q), 4.42(1H,m), 4.57(1H,d), 6.7–7.1(7H,m). ^{1}H-NMR of **III** ($CDCl_3$) δ(ppm), 1.17(3H,t), 1.18-(3H,t), 1.45(3H,t), 3.3–3.6(4H,m), 3.62(1H,dd), 3.74(3H,s), 3.75(1H,dd), 3.86(3H,s), 4.07(2H,q), 4.46(1H,d), 5.22(1H,m), 5.90(2H,d), 6.8–6.9-(3H,m), 7.6–7.9(2H). The chemical structures of these compounds are shown in figs 1 and 2.

2.2. *Enzyme*

Lignin peroxidase of *P. chrysosporium* Burds. (ME-446) was a generous gift from Nagase Biochemicals Ltd (Fukuchiyama, Kyoto), which was prepared and assayed as described in [1].

2.3. *Enzymatic reactions*

The reaction mixture (1.1 ml) contained 20 μl of 25 mM H_2O_2, 0.3 μmol substrate dissolved in 10 μl methanol, 15 μl lignin peroxidase (0.07 IU) and 1055 μl of 100 mM sodium tartrate buffer (pH 3.0). Reaction vessels containing buffer, substrate and H_2O_2 were evacuated and flushed with N_2. The procedure was repeated twice, and finally re-evacuation performed. Then, $^{18}O_2$ (CEA, ^{18}O: 98.58%) was injected into the evacuated vessel. The reaction was initiated by the addition of enzyme and the reaction mixture was incubated at 37°C for 5 min. The reaction was terminated by extraction with ethyl acetate (10 ml). The ethyl acetate layer was washed with saturated NaCl solution (3 ml), dried over anhydrous Na_2SO_4 and evaporated. Half of the extract was reincubated in the medium without addition of enzyme under $H_2{}^{16}O/{}^{16}O_2$ as above. Both the products in the incubation under $^{18}O_2$ and the reincubation were analyzed by GC-MS immediately after evaporation or after acetylation (Ac_2O/pyridine, 1:1; room temperature, 10 h).

Incubation of **I**-Et under $H_2{}^{18}O$ was performed as described (^{18}O content in $H_2{}^{18}O$ of the medium: 49 atom%) [2].

2.4. *Instruments*

^{1}H-NMR and GC-MS spectra were recorded as described previously except for the column [chemical-bonded fused silica capillary column HiCap CBP1-W12-100 (non-polar methyl silicone polymer, 12 m × 0.53 mm (i.d.), Shimadzu, Japan), column temperature: 170–240°C (5°C/min), He: 20 ml/min] [1,2].

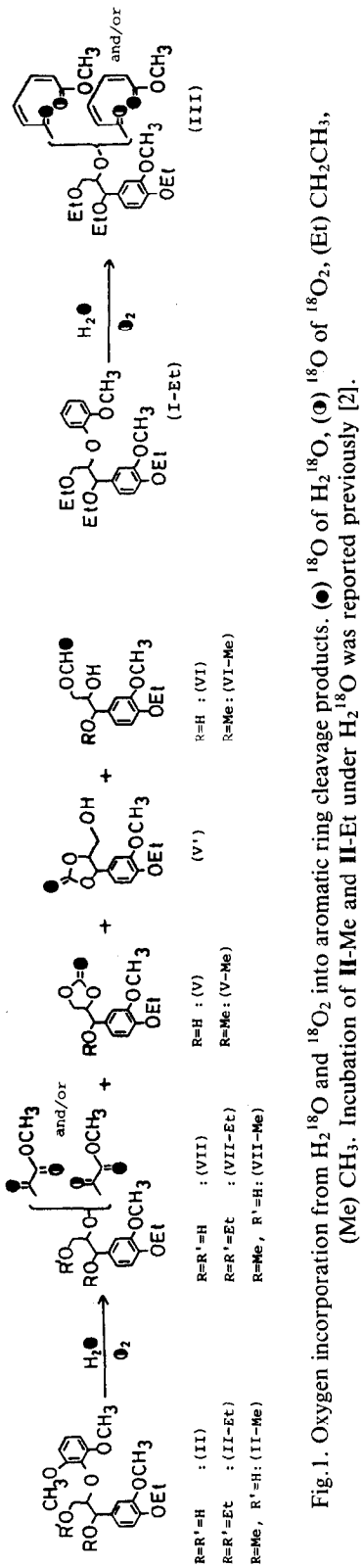

Fig.1. Oxygen incorporation from $H_2{}^{18}O$ and $^{18}O_2$ into aromatic ring cleavage products. (●) ^{18}O of $H_2{}^{18}O$, (◑) ^{18}O of $^{18}O_2$, (Et) CH_2CH_3, (Me) CH_3. Incubation of **II**-Me and **II**-Et under $H_2{}^{18}O$ was reported previously [2].

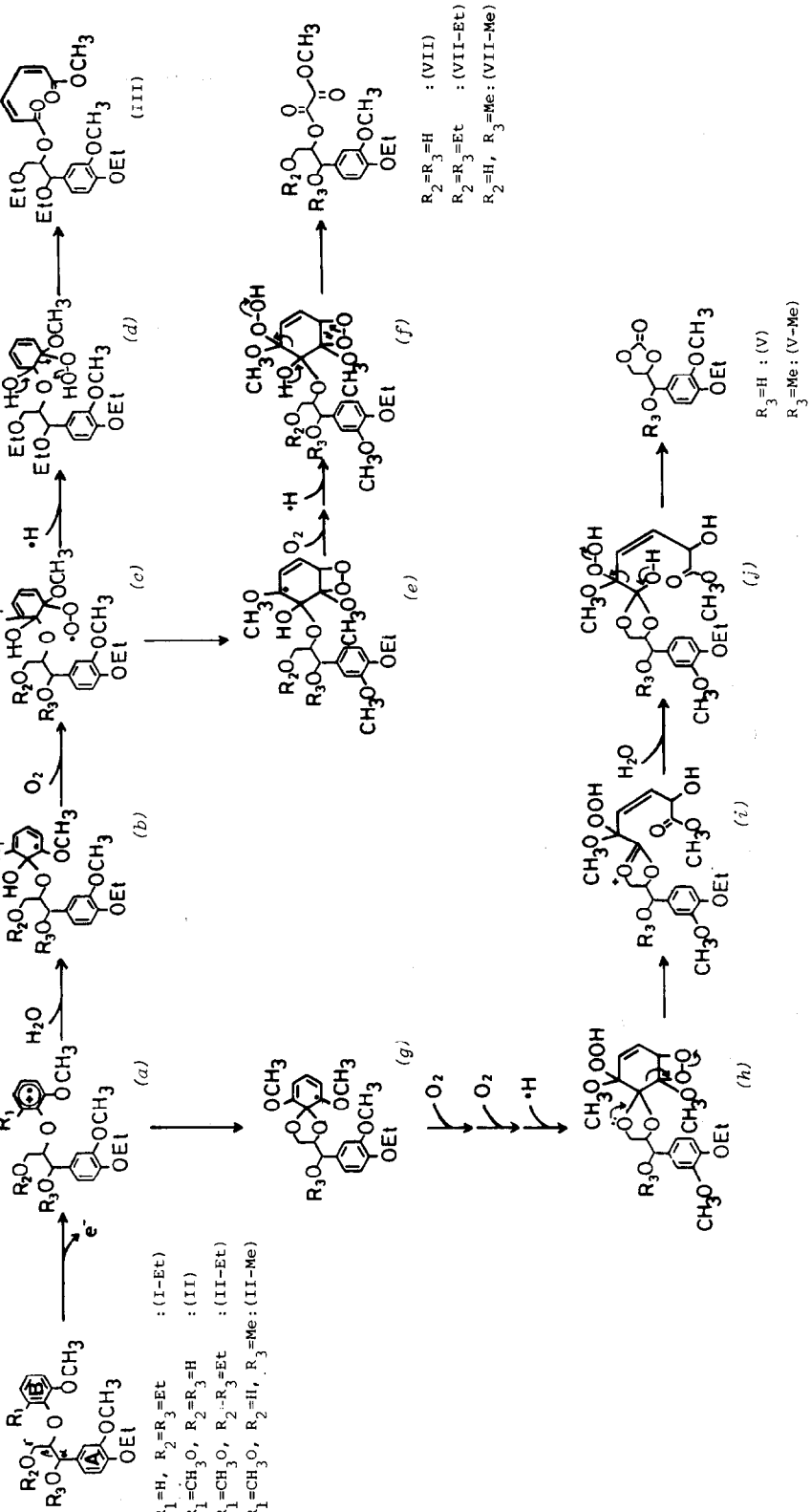

Fig.2. Possible mechanisms for the formation of aromatic ring cleavage products from β-*O*-4 lignin substructure model compounds, **I**-Et, **II**, **II**-Et and **II**-Me. Formation of the α,β-cyclic carbonate **V**′ can be explained in the same way as for **V** and **V**-Me except for nucleophilic attack of CαOH of (a) instead of CγOH.

Table 1

Relative intensities of molecular ion region of mass spectra of products formed on enzymatic oxidation of *β-O*-4 lignin models

a. Muconate **III** formed from **I**-Et

	Relative intensities (%)				
m/z	$H_2{}^{18}O$[a]	$H_2{}^{18}O/H_2{}^{16}O$[b]	$^{18}O_2$[c]	$^{18}O_2/H_2{}^{16}O$[d]	Authentic[e]
434	0	0	4.9	4.9	0
436[g]	91.4	89.1	15.2	12.2	100
438	100	100	100	100	5.0
440	5.2	1.8	12.3	23.2	0

b. Oxalate **VII**-Et formed from **II**-Et

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
382	0	0	0
384[g]	20.7	36.4	100
386	100	100	3.7
388	21.4	27.1	0

c. Oxalate **VII** formed from **II**

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
410	0	0	0
412[g]	11.8	11.1	100
414	100	100	6.5
416	5.1	5.3	0

d. Oxalate **VII**-Me formed from **II**-Me

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
382	0	0	0
384[g]	36.6	32.3	100
386	100	100	7.2
388	7.6	0	0

e. Cyclic carbonate **V** formed from **II**

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
308	0	0	0
310[g]	100	100	100
312	4.0	3.9	2.6

f. Cyclic carbonate **V′** formed from **II**

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
308	0	0	0
310[g]	100	100	100
312	6.2	7.3	2.9

g. Cyclic carbonate **V**-Me formed from **II**-Me

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
280	9.7 (1.4)[f]	11.9 (0)[f]	0
282[g]	100 (100)	100 (100)	100
284	4.6 (3.0)	6.0 (4.2)	2.6

h. Formate **VI** formed from **II**

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
352	0	0	0
354[g]	100	100	100
356	3.5	2.8	2.9

i. Formate **VI**-Me formed from **II**-Me

	Relative intensities (%)		
m/z	$^{18}O_2$	$^{18}O_2/H_2{}^{16}O$	Authentic
324	0	0	0
326[g]	100	100	100
328	2.7	2.4	4.5

3. RESULTS

Identification of all the products except for muconate **III** in degradation of the substrates by lignin peroxidase was reported previously [1,2]. **III** was identified by comparison of the mass spectrum [*m/z* (%): 436(M^+, 1.3), 210(16.5), 209(100), 181(13.7), 153(7.0), 151(15.5), 149(8.5), 125(7.7), 111(5.8), 93(10.8)] and the retention time on GC with those of the synthesized compound; details will be reported elsewhere (submitted).

The results of incorporation of ^{18}O from $H_2{}^{18}O$ and $^{18}O_2$ are shown in table 1 which is summarized as follows (fig.1).

In the formation of muconate **III** from **I**-Et, one atom of ^{18}O was incorporated into the carbonyl groups of the muconate **III** from $H_2{}^{18}O$, and another atom of ^{18}O from $^{18}O_2$.

One atom of ^{18}O was incorporated into the carbonyl groups of methyl oxalates of arylglycerols **VII**-Et, **VII** and **VII**-Me from $^{18}O_2$ in the degradation of **II**, **II**-Me and **II**-Et. Incorporation of ^{18}O into the products from $H_2{}^{18}O$ was reported in [2]. (Incubation of **II** under $H_2{}^{18}O$ was not performed.)

^{18}O incorporation into cyclic carbonates of arylglycerols, **V**, **V'** and **V**-Me was not found in incubation of **II** and **II**-Me under $^{18}O_2$ with the enzyme. On the other hand, one atom of ^{18}O was incorporated into the carbonyl oxygen of **V**-Me in incubation of **II**-Me under $H_2{}^{18}O$, which was reported previously [2].

In the incubation of **II** and **II**-Me under $^{18}O_2$ with the enzyme, ^{18}O incorporation into the formates **VI** and **VI**-Me was not found.

4. DISCUSSION

Based on the previous [2] and present results of the incorporation of ^{18}O from both $H_2{}^{18}O$ and $^{18}O_2$ into the aromatic ring cleavage products (fig.1), we propose the following mechanisms for aromatic ring cleavage of *β-O*-4 lignin substructure model dimers by the lignin peroxidase/H_2O_2 system to give muconate, oxalates and cyclic carbonates of arylglycerols (fig.2). Lignin peroxidase is known to produce cation radicals from methoxylated benzenes including lignin substructure model dimers [8,9]. The mechanisms involve one-electron oxidation of the aromatic ring to the corresponding cation radical [substrate⟶(a)], followed by attack of a nucleophile (H_2O or the hydroxyl groups of Cα and Cγ positions of the propyl side chain) [(a)⟶(b), (a)⟶(g)], and coupling with O_2 [(b)⟶(c), (e)⟶(f), (g)⟶(h)]. Intramolecular addition of peroxy radicals to double bonds to form cyclic peroxides and subsequent coupling with O_2 were demonstrated by Porter et al. [10]. The mechanisms of aromatic ring cleavage by lignin peroxidase proposed here are completely different from conventional ring cleavage of aromatic compounds catalyzed by dioxygenases [12]. A recent study [3] showed that ring cleavage of a *β-O*-4 lignin model by the enzyme occurred only in the presence of O_2 which is in accord with the mechanisms shown in fig.2, and a radical analogous to (g) (fig.2) was suggested for the formation of cyclic carbonates analogous to (**V**). Here, the mechanisms involving nucleophilic attack by H_2O on the cation radicals have been proposed for the first time for aromatic ring cleavage by the enzyme.

Instead of O_2, other radicals derived from O_2 might be involved (e.g. the coupling of (b) with hydroperoxy radical, which was suggested to be formed from O_2 during oxidation of lignin model compounds by the enzyme [12,13]). Since the present result demonstrates the incorporation of ^{18}O from $H_2{}^{18}O$ into the muconate and oxalates, coupling of the cation radical (a) with hydroperoxy

[a,b] Incubation product under $H_2{}^{18}O$ and the product of its reincubation under $H_2{}^{16}O$, respectively (^{18}O content in $H_2{}^{18}O$ of the medium: 49 atom%)

[c,d] Incubation product under $^{18}O_2$ (^{18}O: 98.58%) and the product of its reincubation under $H_2{}^{16}O$, respectively

[e] Unlabeled authentic sample

[f] Analyzed after acetylation

[g] Molecular ion of unlabeled authentic sample

VII, **VII**-Me, **V**, **V'**, **VI** and **VI**-Me were analyzed after acetylation

radical to give a dioxetane is not essential in their formation, which was assumed recently [13]. The mechanism of formation of formates **VI** and **VI**-Me was not fully examined in the present investigation. However, the formyl proton of **VI** was found to be derived quantitatively from H_2O by a tracer experiment using ^2H_2O (not shown). Leisola et al. [4] reported muconate derivatives as ring cleavage products of veratryl alcohol by the enzyme [4]. A mechanism similar to that of the formation of muconate **III** was also proposed for the ring cleavage of veratryl alcohol (Shimada et al., submitted).

Earlier studies indicated that lignin peroxidase catalyzes other degradative reactions of lignin substructure models, which have been reasonably explained on the basis of initial formation of aryl cation radicals [9,12,14,15] as in the aromatic ring cleavage shown in fig.2. Thus, these cation radicals undergo a variety of reactions, such as aromatic ring cleavage, $O-C_4$ cleavage and $C\alpha-C\beta$ cleavage, probably without involvement of enzymes. The mode of the reactions is therefore less specific, and is regarded as being auto-oxidative rather than physiological metabolic pathways involving a series of highly specific enzymatic reactions.

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